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Research report

Interruptions of early cortical development affect limbic association areas and social behaviour in rats; possible relevance for neurodevelopmental disorders

L.M. Talamini ^{a,*}, T. Koch ^a, P.G.M. Luiten ^b, J.M. Koolhaas ^b, J. Korf ^a^a *Department of Biological Psychiatry, University Hospital of Groningen, Groningen, Netherlands*^b *Department of Animal Physiology, University of Groningen, Groningen, Netherlands*

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Abstract

Deficits in social behaviour are found in several neuropsychiatric disorders with a presumed developmental origin. Adequate social behaviour may rely importantly on the associative integration of new stimuli with previously stored, related information. The limbic allocortex, in particular the entorhinal region, is thought to support this kind of processing. Therefore, in the present study, gestating dams were treated with methylazoxymethanol acetate (MAM) on one of gestational days nine to twelve, to interrupt neuronal proliferation in the entorhinal region of the developing fetuses. Effects of prenatal MAM administration on social behaviour were evaluated in adult animals. As the entorhinal cortex has been implicated by some studies in spatial memory, effects on this function were also investigated. Following the behavioural studies, brain morphology was screened for effects of MAM. Our results show moderate to severe social impairment in MAM-treated animals, depending on the exact timing of prenatal exposure. By contrast, spatial reference and working memory were not importantly affected in any group. Analysis of brain morphology in the MAM-treated offspring supported maldevelopment of the entorhinal cortex and revealed mild abnormalities also in some connected limbic and limbic affiliated structures, such as the perirhinal and entorhinal cortex, the anterior cingulate cortex and the medial septum-diagonal band region. Findings are discussed with respect to entorhinal cortex function, and with regard to their relevance for psychiatric disorders with a putatively neurodevelopmental pathogenesis, such as schizophrenia. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Development; Entorhinal cortex; Spatial memory; Social behaviour; Rat; Schizophrenia

1. Introduction

Inadequate social behaviour is a notable characteristic of several putatively developmental psychiatric disorders, including schizophrenia. It has been argued that social impairment in schizophrenic subjects may derive from an inability to make plausible presumptions about the motives of other people's actions [21]. Such inability may be part of a more fundamental impairment, regarding the comparison of new information in general with contextually relevant memory traces. The entorhinal cortex, a supramodal association area in the mediotemporal lobe, conveys highly processed information between neocortical association areas and the hippocampus and is thought to participate importantly in the aforementioned comparative functions [34]. Malfunction of this structure may lead to misinterpre-

tation of social stimuli as well as other types of complex information [34,51].

There are a few reports concerning entorhinal function in humans: a recent PET study, in normal subjects, demonstrated specific engagement of the entorhinal cortex in associative memory [29], while in a number of neuropsychiatric disorders entorhinal cortex pathology is accompanied by cognitive impairment [10]. In schizophrenia, entorhinal cortex abnormalities consist in reduced size and cell number [12,18,24] and disorganised cytoarchitecture (Refs. [1,4,26] but see also Ref. [32]). In addition, other limbic and association areas have been implicated in this disorder, such as the hippocampus, anterior cingulate cortex, prefrontal cortex and parts of the temporal neocortex (for review, see Ref. [51]). Some of the reported neuropathology suggests a prenatal origin, which concords with the absence of gliotic or neurodegenerative signs in schizophrenic brains [43]. Furthermore, various congenital risk factors for the disorder have been demonstrated in epidemiological studies [15,16,47].

* Corresponding author. Department of Psychonomics, University of Amsterdam, Roeterstraat 15, 1018 WB Amsterdam, The Netherlands. Fax: +31-20-6391656; e-mail: talamini@psy.uva.nl

In view of the above, the present study aims to investigate, whether a disturbance of entorhinal cortex development can result in social impairments in an animal model. For this purpose, early stages of cerebrocortical cell proliferation in the rat embryo were interrupted, using methylazoxymethanol acetate (MAM). MAM is a short acting, alkylating agent that permeates the placenta and leads to the death of cells that are actively replicating DNA [35]. This substance was administered to gestating dams on one of four subsequent days of embryonic development (E9 to E12). According to developmental studies, the entorhinal cortex undergoes major cell proliferation during this period [6,7].

Effects of prenatal MAM administration on social behaviour were tested in adult males, in three different social situations: during interaction with an oestrous female, with a subordinate male and with an aggressive male in a resident–intruder paradigm [30]. The priming effect on behaviour of the latter interaction was evaluated 3 weeks after the first exposure, during a second confrontation with the aggressive male. As the entorhinal region has been implicated in the regulation of spatial memory [22,27,28], this function was also studied, to evaluate the specificity of any social impairments. Spatial learning was tested using a hole board set-up [39], which permits one to measure reference memory, as well as working memory [40].

Following the behavioural experiments, morphological effects of MAM were investigated in the entorhinal cortex, but also in other limbic and limbic affiliated regions of the brain. Most of these regions develop precociously and, hence, in relative isolation of other forebrain areas [6,7,54]. Specifically, evaluated regions were the entorhinal, perirhinal and ectorhinal cortices, the hippocampus, the cingulate cortex and various septal nuclei. A more extensive screening of MAM-induced brain abnormalities has been presented elsewhere [54]. Possible relations between behavioural and brain morphological abnormalities were evaluated through correlation analysis.

2. Materials and methods

2.1. Animals and housing

Gestating Wistar WI rats were obtained from Charles River (Germany), who mated animals over a 4-h period, on the day considered as day 0 of gestation. Dams with a vaginal plug were separated from the males and transported to our laboratory where they were housed individually in perspex, sawdust-bedded cages, under standard controlled conditions. Regular rat chow (Hope farms, Woerden, the Netherlands) and tap water, were available *ad libitum*.

Following an intraperitoneal (i.p.) injection (20 mg/kg b.wt.) in pregnant rats, the reaction of MAM with nucleic acids of foetal brain lasts from 2 to 24 h after the injection

and is maximal approximately 12 h post injection [35]. In the present study, eight pregnant dams were injected i.p. with MAM (20 mg/kg b.wt., in NaCl 0.9%) on E9, E10, E11, or E12. Two dams were injected i.p. with saline 0.9% on E10, or E11, to obtain a control group. On the day subsequent to MAM, or saline administration, dams were injected with bromodeoxyuridine (100 mg/kg b.wt., in NaCl 0.9%) for the purpose of histological study. At the adopted dosage, bromodeoxyuridine should not importantly affect cell proliferation and differentiation [37]. The progeny were born on day 22, or 23 of gestation and were reduced to a maximum of 9 pups per litter by eliminating some of the female offspring. Post-weaning male pups were separated from the mother and female siblings and group housed. A maximum of four pups per nest was selected for behavioural testing at the adult age. The experimental groups in the various tests were composed of 5 to 8 male rats, derived from two similarly treated mothers. The MAM-treated groups will be referred to as group E9, group E10, group E11 and group E12. Between different tests, a recuperation period of at least 14 days was intermitted. Rats were handled twice daily, for at least five days, previous to behavioural testing. Only the social interactions were performed under a reversed light/dark cycle (light on 20.00, light off 8.00).

All efforts were made to minimise animal suffering and reduce the number of animals used. The experiments were approved by the local animal welfare committee (FDC1045).

2.2. Hole board learning

At 3 months of age, spatial memory of the animals was tested in a hole board paradigm [39]. The test apparatus consisted of a square arena (70 × 70 × 45 cm) made of PVC. On one of the sides a start box provided access to the arena through a guillotine door. In the floor were 16 equidistant holes, permanently containing a small pellet of white chocolate under a perforated false bottom. Rats were thus prevented to discriminate between baited and non-baited holes by smell. The hole board was placed in a dimly lit room, containing landmarks for orientation, which were kept at fixed positions. Over a period of 1 week, the animals were introduced to the test field, in which all holes were baited. After the introduction period, a 9-day test period began, during which rats had to learn the spatial location of a fixed pattern of four baited holes. The rat was placed in the start box. After 10 s, the guillotine door was lifted. When animals had not left the start box within 2 min, they were gently pushed into the board. There were two trials per day, lasting until the rat had visited all the baited holes, or until 3 min had expired. Results of the morning and afternoon trial were averaged.

A standard event recorder was used to register the visited holes. Hereby, the insertion of the snout into a hole was regarded as a ‘visit’. The following measures were

calculated from the data: Reference memory (food dips + food redips/food dips + food redips + non-food dips + non-food redips), working memory (food dips/food dips + food redips), start box latency (time between opening of the guillotine door and entrance into the hole board), trial duration (time between entrance in the hole board and the last dip) and the average time between dips (time between first and last dip/total dips and redips – 1). Statistical analysis of the data was carried out with repeated measures analysis of variance, comparing each MAM-treated group to the control group (CTRL: 7, E9: 7, E10: 8, E11: 5, E12: 6). The procedures included a between-subjects factor for experimental group and a within-subjects factor for test-day. *T*-tests (for reference and working memory) and the Mann–Whitney *U*-test (for start box latency, trial duration and time between dips) were used for further analysis of significant effects.

2.3. Social interactions

The Wistar rat does not display its full range of aggressive responses until after approximately 6 months of life. Our animals were tested, at 7 months of age, in four types of social interaction: (a) With a receptive female rat, in neutral territory, during 10 min. (b) With a younger, male opponent, approximately 10% lighter in body weight, in neutral territory, during 10 min. (c) With a territorially aggressive, resident male, in a resident–intruder paradigm, during 10 min, or until complete submission of the experimental rat. (d) During a second confrontation with the same aggressive resident, 3 weeks after the first encounter (3 min).

For the sexual interactions 12 female, Wistar rats (Harlan) were sterilised by ligation of the oviducts and brought to oestrus by injections of oestrogen and progesterone. The lighter male opponents were naive, group-housed Wistar rats (Harlan), while Tryon Maze Dull (TMD) S3 rats of 4–6 months of age (originally derived from Cpb, TNO, Zeist, The Netherlands and bred at the Biological Centre in Haren) were used as resident males. TMD S3 males are known as spontaneously, territorially aggressive animals. They were ‘permanently’ housed in wooden observation cages (85 × 60 × 50 cm) with a sterilised female, which was removed during interaction experiments. During the second confrontation tests, the TMD S3 males were constrained in a small, wire netting cage within the home cage, to prevent them from actually attacking the experimental rats. For a more detailed description of the resident–intruder paradigm see, e.g., Ref. [30].

During each social interaction, various behavioural elements were recorded that together account for all, or nearly all, behaviour in an observation period. The behavioural elements listed hereafter, have been described and discussed in more detail by others [9,23,30,33].

Interaction with the female: exploring (moving around the cage, scanning, or sniffing the air, walls or floor),

rearing (vertical posture of the body with both forepaws raised or placed against the walls), grooming (self-grooming: keeping the mouth and paws on the body or on the head), immobility (absence of movements, with or without signs of fear), moving away from the female (moving away from a position of close proximity to the partner), moving toward the female (moving directly toward the female after a period of non-interaction), following the female (both rats are moving around the cage, the male closely behind the female, often maintaining a nose contact with her anogenital area), investigating (sniffing the partner, except the anogenital region), social grooming (nibbling, wiping, or biting the fur of the partner, in particular near the head and the dorsal region), genital sniffing (sniffing the anogenital region of the partner), mount attempt (unsuccessfully mounting the partner, most often because the partner moves away), mounting (male climbs on to rear of female with rapid pelvic thrusting), penis grooming (self-grooming of genital area shortly following mounting).

Interaction with the subordinate male: exploring, rearing, grooming, immobility, investigating, social grooming, genital sniffing, lateral threat (slowly moving in a sideways direction to V-wards, or around the opponent; piloerection, arched back, body close to the substrate. The animal may keep distance but may also push away its opponent), upright (the rat stands on its hindlegs in reaction to approach, or upright of the opponent; piloerection in the dominant male, eyes may be half closed. The rats may hold on to each other’s forepaws), chasing (running towards, or following the opponent at high speed), clinching (very rapid rolling, jumping and biting of both animals that are in close contact), keeping down (standing over the opponent; pinning it down against the substrate), keeping off (kicking with hindleg towards the opponent; standing position), mounting (mounting the opponent as in a male–female encounter).

Interaction with the aggressive resident: exploring, rearing, grooming, investigating, immobility, submitting (lying on the back during and following keeping down by the opponent), fleeing (moving away from the opponent at high running speed), upright, lateral threat, keeping down, clinching, chasing, moving toward, moving away.

Second confrontation with the aggressive resident: exploring, rearing, grooming, investigating (sniffing the opponent, or the wire netting cage containing it), immobility, fleeing, lateral threat, moving toward (the cage), moving away (from the cage).

Behaviour was recorded during the dark phase of the light–dark cycle, using an event recorder. The test room was lit dimly with red light. Behavioural elements, expressed as frequencies and durations, were analysed individually, but also grouped into behavioural categories by linear summation, as shown in Table 1. In view of the varying predominance of different behavioural elements in the rats behaviour, care was taken to avoid categories that

Table 1
Categories of behavioural elements during various social interactions

Behavioural category	Constituting behavioural elements
<i>Wistar female</i>	
Introductory behaviour	Investigate, genital sniff, social groom, move toward, follow
Sexual behaviour	Mount attempt, mount penis groom
Non-social behaviour	Explore, rear, groom
<i>Wistar male</i>	
Introductory behaviour	Investigate, genital sniff, social groom
Non-social behaviour	Explore, rear, groom
<i>TMD S3 male</i>	
Agonistic behaviour	Lateral threat, upright, chase, keep down, move toward, investigate
Defensive behaviour	Upright, move away, flee, immobile, submit
Non-social behaviour	Explore, rear, groom
<i>Social recall</i>	
Non-social behaviour	Explore, rear, groom

would largely reflect only one, frequently displayed, behavioural element. Data were analysed by ANOVA for each recorded behavioural element, considering prenatal treatment as between-subjects variable (CTRL: 5, E9: 5, E10: 6, E11: 5, E12: 6). In addition, repeated measures analyses of variance were conducted for each behavioural element, with prenatal MAM treatment as between subjects variable and the sequence of social interactions (with a subordinate male, a female and twice with the same S3 male) as within-subjects variable. The behavioural categories were only analysed through one way ANOVA, as they did not contain exactly the same behavioural elements in different interactions. Post-hoc comparisons were performed through simple contrast analysis. Thereby, the prenatally saline injected animals were treated as the reference group and all other groups were compared against it.

2.4. Morphological screening

Following behavioural tests, animals were brought under deep pentobarbital anaesthesia and were transcardially perfused with 0.1 M PBS pH 7.4, followed by 300 ml 4% paraformaldehyde in 0.1 M PBS. The brains were removed from the skull, carefully dried with absorption paper, weighed and processed for immunohistochemistry against PKC- γ (monoclonal 36G9, Chemunex) following standard procedures, described in detail previously [54]. Alternating sections were processed for Nissl staining.

In the immunohistochemically stained coronal sections (30 μ m), the following measures were taken: Length of the medial entorhinal cortex (MEC), length and thickness of the lateral entorhinal cortex, posterior part (LEP) and anterior part (LEA), thickness of the perirhinal (PRC) and entorhinal cortex (ECT). Coronal surface area of the entire

entorhinal cortex was measured at a relatively posterior rostrocaudal level. In addition, length of the pyramidal layer of the hippocampal Ammon's horn was determined at an anterior and posterior level, whereby CA1/CA2 and CA3/CA4 were measured separately. Furthermore, length of the cingulate cortex was determined at four rostrocaudal levels. Cortical length was measured along the most superficial cell layers, while measures of cortical thickness encompassed only the cellular layers (II–VI).

The acetylcholine esterase stained sections were used for areal measurements of the lateral septal nucleus, septo-hippocampal nucleus and medial septal nucleus/horizontal limb of the diagonal band (MS-DB). As the medial septal nucleus and diagonal band (or medial septal complex) are not clearly distinguishable, the width of the horizontal limb of the diagonal band (DB) was determined, in a linear measure perpendicular to its longitudinal axis, to obtain a separate measure of this structure.

The differentiation between various brain regions was based on morphological and cytoarchitectural criteria [25,58]. The coronal planes of measurement were carefully selected, using sets of specific criteria for each measured region. Morphometry was assisted by a computerised image analysis system. The data was analysed through Mann–Whitney *U*-tests, to determine mean differences between the control group and the various MAM-treated groups.

2.5. Correlation analysis

Spearman correlation coefficients were calculated for behavioural and brain morphological variables that were significantly affected by prenatal MAM administration. In view of the multiple tests, only correlation coefficients larger than 0.5, reaching *p*-values of 0.01, or less, were considered statistically significant. We chose not to adopt a Bonferroni, or similar correction to maintain an acceptable level of power in the statistical procedure.

3. Results

3.1. Hole board learning

No main effects of MAM treatment were found either in reference memory (Fig. 1A), or in working memory. Repeated measures analyses did, however, indicate a prenatal treatment by day effect for working memory in the comparison of group E10 vs. control ($F = 5.9$, *df* 8, 104, $p < 0.02$; Fig. 1B). Post-hoc tests revealed a significantly decreased working memory mean, with respect to the control group, on the fifth test day only ($p < 0.05$).

A number of non-memory measures were also calculated from the data: Trial duration, a 'time between dips'

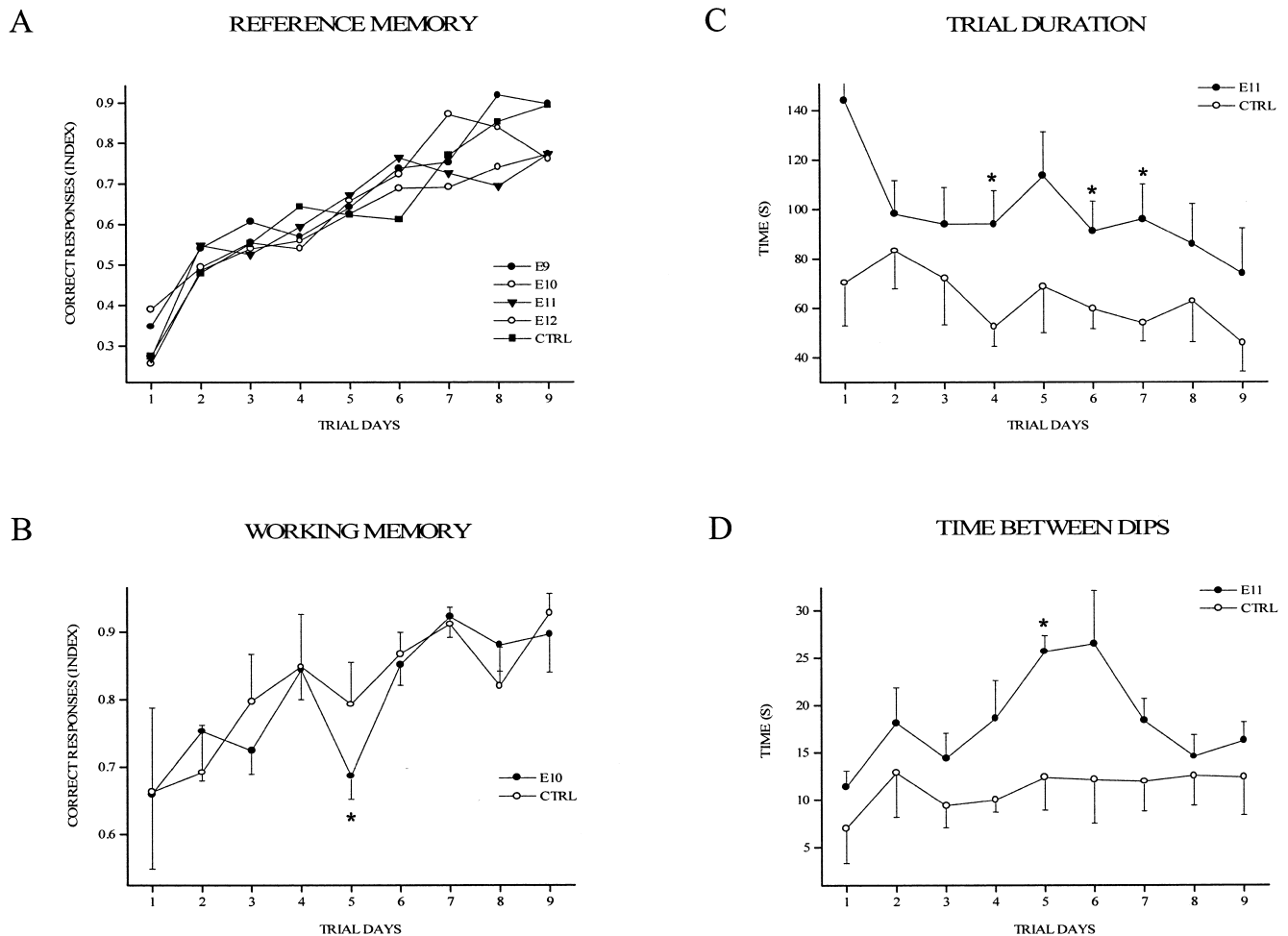


Fig. 1. Effects of prenatal MAM administration on memory and non-memory parameters, in a hole board learning experiment. Reference memory is not affected in any MAM-treated group (A). B, C and D only show the groups that differ significantly from control: There is a repeated measures 'treatment by day' effect for working memory, in the comparison of the E10-treated group vs. control (B). A similar interaction effect emerges for the average time between hole visits, in the comparison of E11-treated rats and controls (C). Trial duration is generally increased in E11-treated rats with respect to control (D). The two interaction effects appear to reflect decreased performance of E10 and E11-treated rats on the fifth learning day, when the weekly cleaning routine of the animal laboratories took place. * $p < 0.05$ in post-hoc tests.

index (indicating the average time that elapses between two subsequent hole visits) and start box latency. In the comparison of group E11 vs. control a number of non-memory parameters were significantly different: repeated measures analysis showed a main effect of treatment in trial duration ($F = 6.7$, df 1, 10, $p < 0.03$; Fig. 1C) and a significant treatment by day interaction in the time between dips ($F = 2.1$, df 8, 80, $p < 0.05$; Fig. 1D); The latter interaction reflects an increased average time between hole visits in E11-treated rats with respect to controls on the fifth learning day (post-hoc tests: $p < 0.01$).

3.2. Social behaviour

3.2.1. Social behaviour in control rats

During interaction with the lighter male and the receptive female, behaviour of control rats centred on social introduction and exploration of the environment. In addition,

with the female and sometimes with the subordinate male, sexual behaviour was displayed.

In the first resident–intruder interaction, behaviour of control, intruder rats would initially resemble behaviour displayed in interaction with the lighter male. However, within the first few minutes of interaction, the threatening attitude of the resident male was perceived and behaviour of the intruder became almost exclusively defensive. Hereby, both active (e.g., upright, keep off) and passive (e.g., immobile, submit) elements of defence would be displayed.

During the second confrontation with the TMD S3 male, behaviour of control intruders clearly showed priming by the first encounter: Hardly any exploratory, or introductory social behaviour was displayed. Instead, a strictly defensive behavioural pattern set in right at the start of interaction and persisted up to the end. As the wire netting cage prevented actual attacks by the resident, the

intruder typically displayed passive elements of defence. In fact, most control animals remained immobile during most of the observation period.

3.2.2. Effects of MAM on four types of social interaction

For the interactions with the lighter Wistar males ANOVA (df 4, 22) indicated a difference between groups in social grooming (frequency, $F = 3.2$, $p = 0.03$; Fig. 2A) and genital sniffing (frequency: $F = 5.9$, $p = 0.002$; duration: $F = 3.9$, $p > 0.02$). According to contrast analysis duration of genital sniffing was significantly decreased in E9 ($p = 0.008$), E11 ($p < 0.02$) and E12 rats ($p = 0.001$) with respect to controls (Fig. 2B); genital sniffing frequency was only significantly decreased in E12-treated rats ($p = 0.002$). Post-hoc comparisons of social grooming were not significant.

In interaction with the females investigating (frequency: $F = 3.7$; duration: $F = 3.3$, $ps < 0.03$) and again social grooming (frequency: $F = 3.6$; duration: $F = 3.1$, $ps < 0.04$) differed significantly between groups. In addition, differences were found in non-social behavioural elements, like the frequency of exploring ($F = 6.8$, $p = 0.001$), rearing ($F = 3.8$, $p < 0.02$) and total non-social behaviour ($F = 3.6$, $p = 0.02$). These findings appear to be largely due to abnormalities in the E12 group. Post-hoc analyses, in fact, showed significant increases in E12-treated rats with respect to the control group, for investigating (frequency: $p < 0.02$, duration: $p = 0.004$; Fig. 3A), social grooming (frequency: $p < 0.03$; Fig. 3B), exploring (frequency: $p < 0.001$; Fig. 3C), rearing (frequency: $p < 0.03$; Fig. 3D) and total non-social behaviour (frequency: $p = 0.005$).

The interaction with the territorially aggressive TMD S3 males revealed differences between the experimental groups in exploring (duration: $F = 3.4$, $p < 0.03$), total non-social behaviour (frequency: $F = 3.0$; duration: $F = 3.6$, $ps < 0.04$) and total agonistic behaviour (frequency: $F = 3.6$; duration: $F = 3.2$, $ps < 0.03$). The latter category contains both aggressive and introductory behavioural ele-

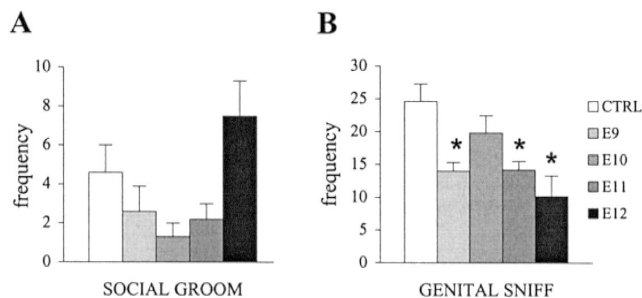


Fig. 2. Effects of prenatal MAM administration on behaviour with a lighter male in neutral territory. Significant ANOVA effects were found in social grooming (A) and genital sniffing (B). The effects appear to be largely due to alterations in behaviour of E12-treated rats. * $p < 0.05$ in post-hoc tests.

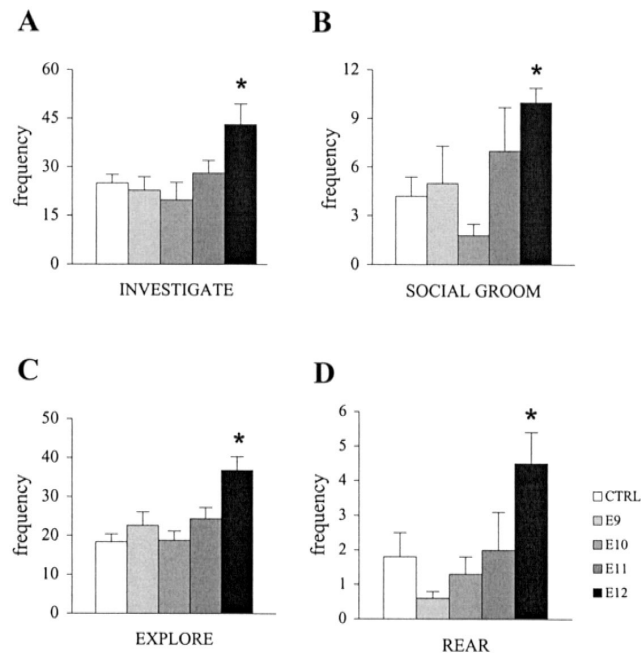


Fig. 3. Effects of prenatal MAM administration on behaviour with a receptive female in neutral territory. Significant ANOVA effects were found in investigation (A), social grooming (B), exploring (C) and rearing (D). The effects appear to be largely due to alterations in behaviour of E12-treated rats. * $p < 0.05$ in post-hoc tests.

ments (Table 1). According to post hoc comparisons, effects in the non-social behavioural elements were again caused by increased occurrence in the E12 group: duration of exploring ($p < 0.02$) and total non-social behaviour (frequency: $p = 0.02$, duration: $p < 0.02$; Fig. 4A) were significantly increased in this group with respect to control rats. By contrast, the ANOVA effect in total agonistic behaviour appears to reflect an increase in E9 (frequency: $p < 0.02$, duration: $p < 0.04$) and E10-treated rats (duration: $p < 0.03$) with respect to control rats (Fig. 4B).

Finally, in the repeated confrontation with the TMD S3 males a significant ANOVA effect was found for investigating (frequency: $F = 3.3$, $p < 0.03$; duration: $F = 5.1$, $p = 0.005$). The post-hoc comparisons indicated significantly increased frequency ($p < 0.02$) and duration ($p = 0.002$) of investigating in E9 rats, in comparison to control animals (Fig. 4C).

3.2.3. Repeated measures analysis over subsequent social interactions

Effects of MAM treatment on social behaviour were also analysed through ANOVA procedures, with prenatal MAM treatment as between subject variable and the different social tests as within subject variable. These procedures incorporate data from all subsequent social tests in which a given behavioural element occurs and analyse whether there is an alteration independent of, or in relation to a specific social context. This approach revealed main effects of prenatal MAM administration on social groom-

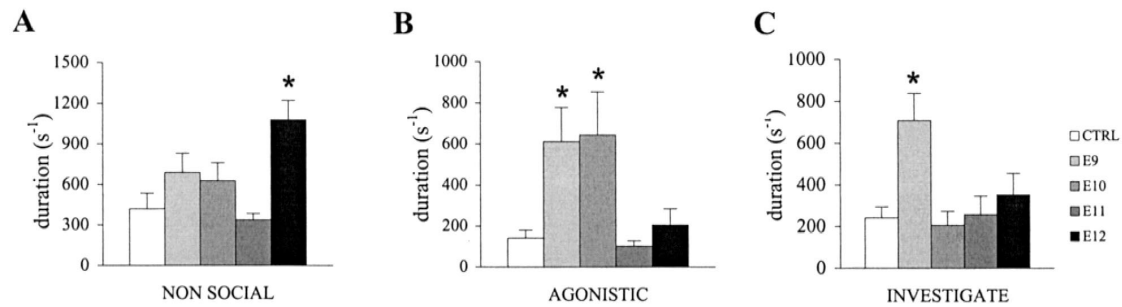


Fig. 4. Effects of prenatal MAM administration on behaviour, during a novel (A,B) and a repeated (C) encounter with a territorially aggressive male in a resident-intruder paradigm. A significant ANOVA effect was found for total non-social behaviour (A), reflecting an increase in E12-treated rats. Further, effects were found in total agonistic behaviour (B) and investigating (D). The latter effects mainly reflect alterations in behaviour of E9-treated rats. * $p < 0.05$ in post-hoc tests.

ing (frequency: $F = 6.39$; duration: $F = 6.35$, df 4, 22, $ps < 0.001$), genital sniffing (frequency: $F = 3.26$, df 4, 22, $p < 0.03$), investigating (duration: $F = 2.97$, df 12, 66, $p < 0.04$) and exploring (frequency: $F = 2.94$, df 12, 66, $p < 0.04$). Thereby, social grooming and genital sniffing were registered only in the two non-threatening interactions, while the other behavioural elements were recorded in all interactions. According to post hoc tests, both E9 and E12-treated rats display significantly increased duration of investigating ($p < 0.02$ and $p = 0.005$, respectively), E12 rats furthermore display increased social grooming (frequency: $p < 0.009$, duration: $p = 0.01$) and explore more often (frequency: $p < 0.02$). Finally, the frequency of genital sniffing is decreased with respect to controls in E11- ($p < 0.05$) and E12-treated rats ($p < 0.02$). The largest deviations from control, in the above parameters, tend to occur in the E12-treated rats.

Superimposed on the main effect in exploring frequency is an interaction effect of treatment and social interaction ($F = 2.3$, $p < 0.02$). Significant contrasts with respect to the control group are found for the E9 group ($p < 0.05$), the E11 animals ($p = 0.02$) and, most strikingly, for E12 rats ($p = 0.0003$).

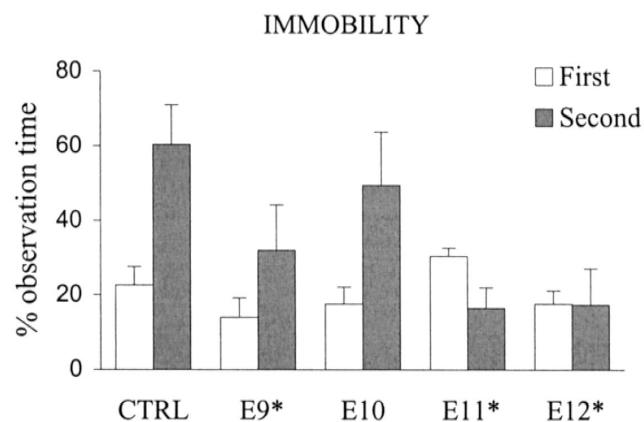


Fig. 5. Immobility data from two subsequent resident-intruder interactions reveal significantly different behavioural profiles in rats treated with MAM on E11 and E12, with respect to control rats. Repeated measures ANOVA interaction effect, $p < 0.05$. * $p < 0.05$ in post hoc tests.

3.2.4. Behavioural priming over two resident-intruder interactions

Another repeated measures analysis compared behaviour over the two sequential interactions with the same territorially aggressive male, revealing an interaction effect for immobility (duration: $F = 5.2$, $p < 0.005$, df 4, 22). A similar effect is found when all types of social encounters are included in the repeated measures ANOVA. However, immobility was typically displayed in interactions with the territorially aggressive males and hardly at all occurred in the two non-threatening social interactions. Post-hoc contrasts indicated that the E9 ($p < 0.04$), E11 ($p = 0.0004$) and E12 ($p < 0.008$) group deviated significantly from control. Indeed, in the control group the time spent in immobility was notably increased from the first to the second interaction with the territorially aggressive male. In E9 animals, this increase was much less pronounced. Strikingly, animals treated on E12 did not display this increase at all, while E11-treated animals even appeared to reduce immobility in the second interaction (Fig. 5). The difference between E9 rats and controls may be related to the agonistic attitude of these rats in interaction with the TMDS3 males.

3.3. Brain morphology

Following prenatal MAM treatment, brain weight (Table 2) was slightly different from control only in groups exposed on E9 (104%, $p < 0.03$) and E12 (94%, $p < 0.02$). However, analyses of brain morphology indicated abnor-

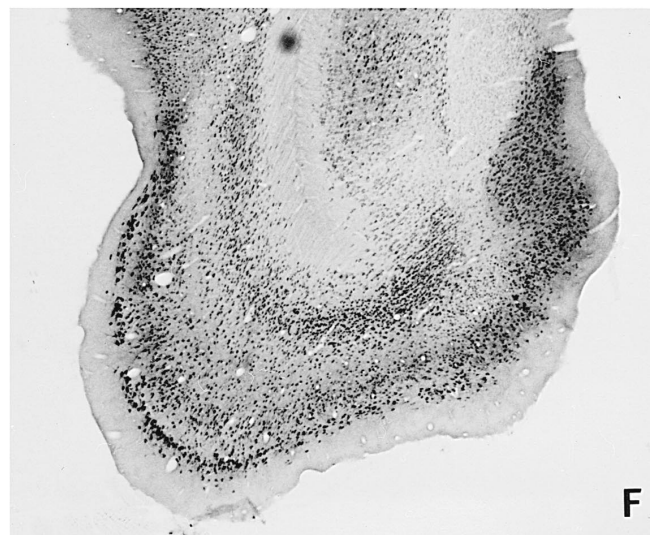
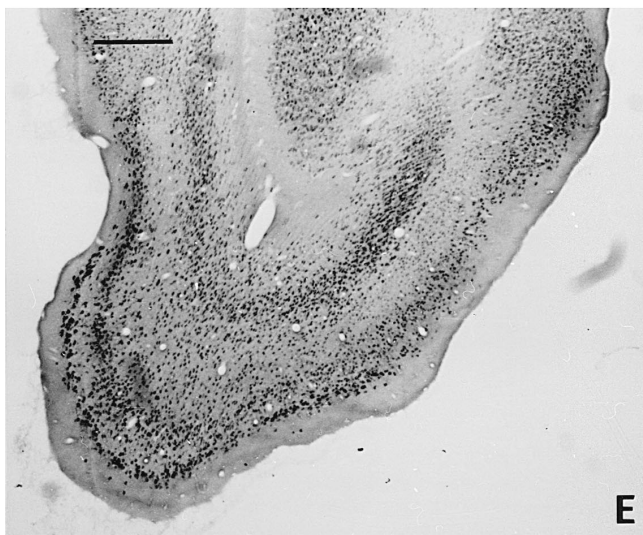
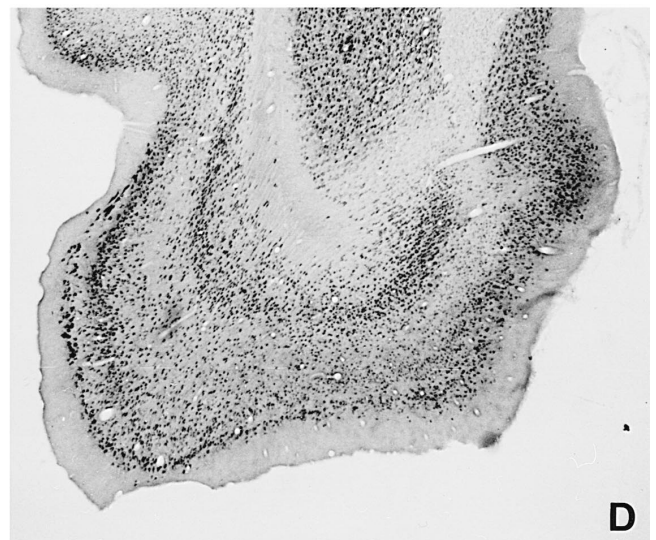
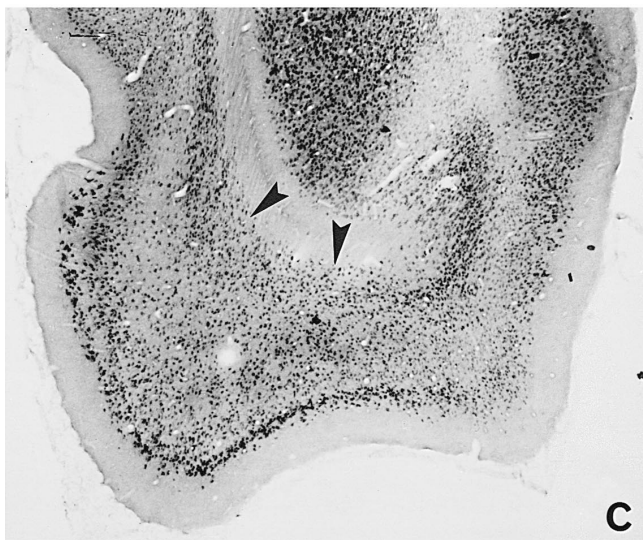
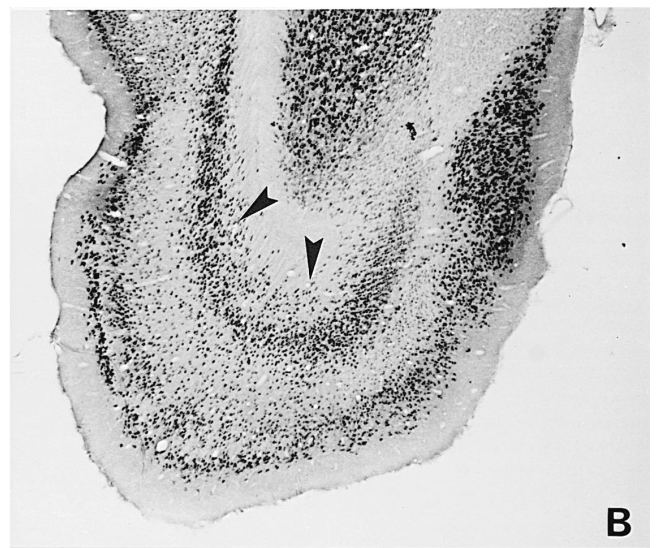
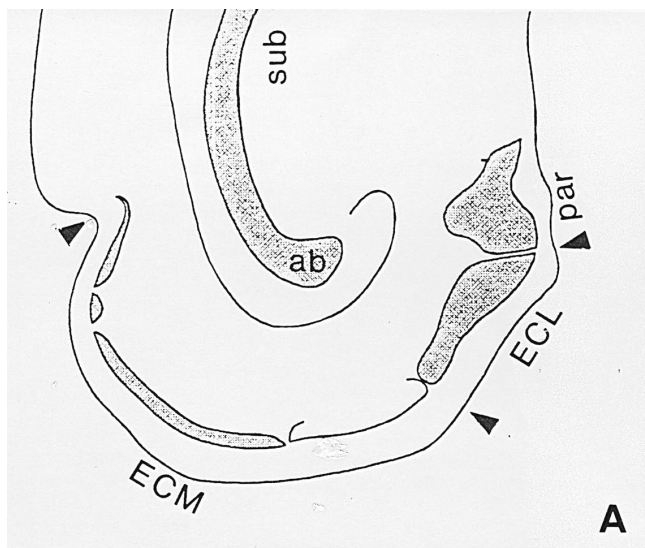
Table 2
Brain weight (mg)

	Mean \pm S.E.M.	% of CTRL
CTRL	2052 \pm 32	100
E9	2134 \pm 19	104*
E10	2121 \pm 38	103
E11	2096 \pm 19	102
E12	1925 \pm 20	94*

* $p < 0.05$.

mal development of the entorhinal cortex in all MAM-treated groups (Fig. 6). In fact, cortical length of the LEP

was significantly reduced in groups E9 (80%, $p < 0.01$), E10 (86%, $p < 0.03$) and E11 (89%, $p < 0.05$; Fig. 7A),



length of the LEA was significantly reduced in group E9 (82%, $p < 0.03$) and E10 (78%, $p < 0.01$; Fig. 7B) and length of the MEC was significantly reduced in group E12 (68%, $p < 0.01$; Fig. 7C). In addition, group E12 displayed significantly reduced cortical thickness in the LEP (84%) and LEA (86%; $ps < 0.05$). As a resultant, entorhinal cortex surface area was reduced in all groups (E9: 85%, E10: 79%, E11: 73%, E12: 74%), reaching statistical significance in groups E11 ($p < 0.03$) and E12 ($p < 0.04$).

Effects of prenatal treatment were also found in the PRC and ECT, which lie dorsally adjacent to the entorhinal cortex, in and over the rhinal fissure, respectively. Cortical thickness of the PRC was significantly reduced in all groups (E9: 82%, E10: 79%, E11: 80%, E12: 80%, $ps < 0.05$), while thickness of the ECT was significantly reduced in groups E9 (89%, $p < 0.05$) and E12 (87%, $p < 0.05$). Further morphological changes were observed in the septal region (Fig. 8), where measurements of coronal surface area demonstrated reduction of the MS-DB in groups E9 (85%, $p < 0.02$), E11 (89%, $p < 0.03$) and E12 (81%, $p < 0.005$; Fig. 9A) and of the SH in group E12 (78%, $p < 0.03$). In addition, width of the DB was significantly decreased in group E9 (84%, $p < 0.03$; Fig. 9B). No significant changes were found in the lateral septal nucleus. As can be seen in Fig. 8, the dorsal delimitation of the septum was progressively reduced in groups E10 to E12, leaving only a thin tissue bridge between the septum and corpus callosum in E12 rats.

Some small, but statistically significant, changes occurred in other limbic regions under investigation. Specifically, length of the anterior cingulate cortex was increased in group E9 (110%, $p < 0.05$), while length of the CA1/CA2 region in the posterior hippocampus was slightly decreased in the E12 group (93%, $p < 0.02$). The coronal area of the posterior hippocampus was also slightly reduced in this group (91%, $p < 0.04$). No consistent changes were found at more posterior levels of the cingulate cortex, or at anterior levels of the hippocampus.

Disorganised cytoarchitecture was observed in several abovementioned regions in MAM-treated rats; most prominently in the entorhinal cortex of E9-exposed rats, where all cortical layers except layer 2a were poorly delineated (Fig. 6C). Moreover, in this group the anterior cingulate region showed thinner layers, with more densely packed PKC- γ stained pyramidal neurons in layers II/III and V (Fig. 10B). In other MAM-treated groups, cytoarchitecture of entorhinal (Fig. 6D–F) and other limbic areas appeared somewhat more untidy than in controls, but individual

cortical layers could generally be distinguished, suggesting lamination to be basically intact.

3.4. Correlations between behavioural and morphological abnormalities

As shown in Table 3, several behavioural elements affected by prenatal MAM treatment correlate significantly with measurements of the limbic cortex and the MS-DB region. Specifically, for behaviours displayed with the female, length of the LEP correlates with investigation (frequency: $\rho = 0.64$; duration: $\rho = 0.60$, $ps < 0.001$), length of the MEC correlates inversely with social grooming (frequency: $\rho = -0.60$, $p < 0.001$; duration: $\rho = -0.53$, $p = 0.002$), thickness of the ECT correlates inversely with investigation (frequency: $\rho = -0.52$, $p = 0.003$), thickness of the LEA correlates inversely with non-social behaviour (frequency: $\rho = -0.51$, $p = 0.004$) and width of the DB correlates with social grooming of the female (duration: $\rho = 0.51$, $p = 0.005$). Furthermore, length of the anterior cingulate cortex correlates inversely with social grooming of the subordinate male (frequency: $\rho = -0.51$; duration: $\rho = -0.56$, $ps < 0.004$), while coronal surface area of the MS-DB correlates inversely with investigation (frequency: $\rho = -0.59$, $p < 0.001$) and agonistic behaviour (frequency: $\rho = -0.58$, $p < 0.001$), during interaction with the territorially aggressive male.

4. Discussion

Results from these experiments show that interference with cell division during early cortical neurogenesis, from E9 to E12, affects maturation of various limbic and limbic affiliated regions of the brain, such as the entorhinal, perirhinal and ectorhinal cortices, the anterior cingulate cortex and the MS-DB region. Hypoplasia of the entorhinal cortex appears to be progressively more severe and to encompass more medial regions with later MAM administration. However, effects on cortical cytoarchitecture were most notably found in E9-treated animals. MAM-exposure at this time may interfere with the formation of Cajal–Retzius and subplate neurons, which are thought to be essential for the development of normal cortical morphology. These cells are formed during the earliest phases of cortical development (for a more extensive discussion of MAM effects on cytoarchitecture see Ref. [54]).

Fig. 6. Representative photomicrographs of PKC- γ immunostained sections, showing the entorhinal cortex of a control rat (B) and of MAM-treated rats from groups E9 to E12 (C–F). Lateral and medial subdivisions of the entorhinal region are schematically illustrated in (A). Reduction of the entorhinal area gets more severe with later MAM treatment. Cortical shortening is largely limited to the lateral subdivision in groups E9 to E11 (C,D,E) and shifts to encompass the medial subdivision in group E12 (F). Notice the loss of layered cortical organisation in the E9 entorhinal cortex (arrows in C) with respect to control (arrows in B), particularly in the deep cortical laminae. Disorganisation appears much less substantial in later treated groups. Abbreviations: ab, angular bundle; ECL, entorhinal cortex lateral subdivision; ECM, entorhinal cortex medial subdivision; par, parasubiculum; sub, subiculum. Bar: 500 μ m.

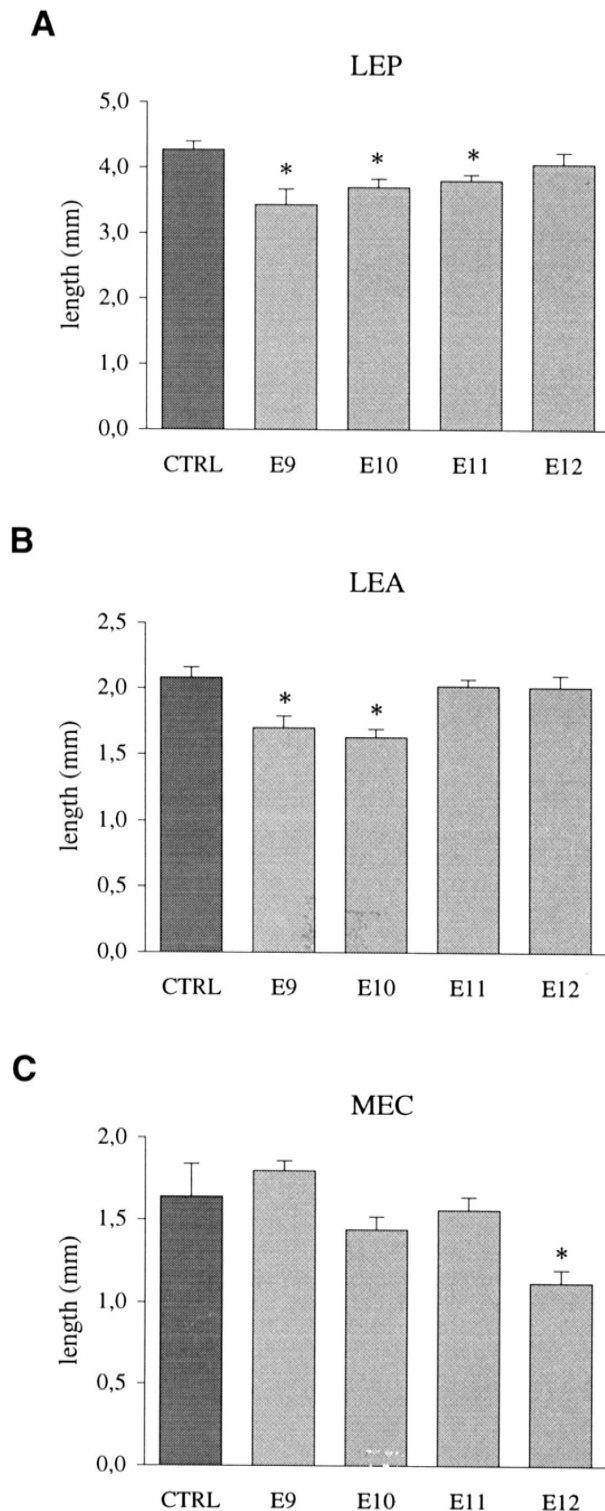


Fig. 7. Mean shortening of the lateral entorhinal cortex at a posterior (A) and a more anterior level (B) following prenatal MAM administration on various days of embryonic development (E9–E12). The medial subdivision of the entorhinal region does not stretch as far rostrally as the lateral division and cortical length was thus only measured at a posterior level, as depicted in (C). * $p < 0.05$ in Mann–Whitney U -tests.

According to a previous morphological study (involving the entire neo- and allocortex, the thalamus and various

parts of the striatum) MAM treatment, as adopted here, produces relatively localised effects on brain morphology [54]. This can be understood considering that at this early stage of development the rat CNS is not undergoing major cell proliferation. Only forebrain regions that originate precociously, among which are the parahippocampal gyrus, MS-DB region, and anterior parts of the cingulate cortex, are hence notably compromised [6,7]. For comparison, at E15, when extensive cell proliferation accompanies formation of the neocortex and various subcortical regions, the same dose of MAM results in pronounced microencephaly [35].

Notably, some regions in the upper hindbrain and brainstem also originate relatively precociously, such as part of the monoaminergic areas [32a] and deep cerebellar nuclei [50a]. Minor morphological effects of MAM in the dopaminergic and serotonergic nuclei were found following treatment on E12, but not with earlier administrations (manuscript in preparation). With respect to other hindbrain and brainstem regions we can at this point not exclude maldevelopment due to MAM, although no macroscopic abnormalities have been observed in these areas. It should in this respect be considered that various factors complicate the prediction of consequences of prenatal insults from developmental timetables. For instance, the relative plasticity of various systems to a proliferative insult might vary. Furthermore, structures that complete neurogenesis in a brief time span may be more sensitive to a proliferative defect than areas that form over a longer period of time.

At the behavioural level, prenatal MAM treatment affected social interaction in adult rats, while leaving spatial learning essentially intact. Effects on social behaviour differed with prenatal exposure day. Rats exposed to MAM at E9 or E10 displayed abnormal agonistic behaviour toward the territorially aggressive TMD S3 males, i.e., they presented significantly more introductory behaviour than other groups and also displayed offensive behaviour such as lateral threats, chasing and keeping the opponent down. The occurrence of the latter patterns was striking, since aggressive behaviour is uncommon in Wistar males and was, indeed, rarely observed in control animals. Our correlation analyses suggest that there might be a relation between these behavioural alterations and reductions of the MS-DB region. Accordingly, various studies have implicated this region in the regulation of aggressive behaviour [2,5].

Further behavioural abnormalities were found with treatment on E11 and E12, which resulted in a reduced priming effect of a stressing social experience on behaviour in a subsequent, similar situation. Considering the results from the hole board experiment this is not likely due to a general memory impairment in these rats. The impairment appears to be subtler, relating perhaps to specific types of information, or isolated aspects of the memory process (acquisition/retrieval/consolidation). In view

of studies by others, we propose that the findings may reflect an impediment in the use of previous social experience in the evaluation of a related situation. Indeed, adult lesions of the entorhinal cortex, or its hippocampal connections were shown to disrupt various behavioural phenomena requiring this type of contextual processing, including latent inhibition [59], contextual modulation of conditioned responses [19] and the capacity to make transitive inferences between information presented on separate occasions [17]. Similarly, tasks wherein taking into account previous information slows down, or hinders performance appear to be facilitated after entorhinal lesions [41,48].

Behavioural effects of prenatal MAM administration were, on the whole, most pronounced in E12-treated animals, which, besides the abovementioned priming deficit, presented with abnormalities in all types of social interaction considered in this study. Behaviour in these rats was characterised by exaggerated occurrence of certain elements of social introduction, such as investigating and grooming the partner, while at the same time non-social, exploratory behaviour was increased. Interestingly, changes in exploratory behaviour and reaction to novelty were also observed in non-social test paradigms, following adult lesions of the MEC in mice [46]. The earlier-mentioned abnormalities were demonstrated both during the non-threatening interactions (with the male and female Wistar opponents) and during the threatening resident-intruder encounters. In the latter context, E12 rats' behaviour was in marked disagreement with the biological relevance of the situation, which was also apparent in unquantified observations. For instance, typical fear related behaviour, like 'rigid' and slow movement, or offence related responses, like piloerection, were infrequently seen in E12-treated animals. During the second resident-intruder interaction a number of animals in the E12 group was even observed to climb the wire netting cage constraining the resident male and extensively groom themselves on top of it. On the whole, observation of these rats suggests severely impaired processing of social stimuli. Such impairment may have contributed to the lack of behavioural priming observed over the two resident-intruder interactions.

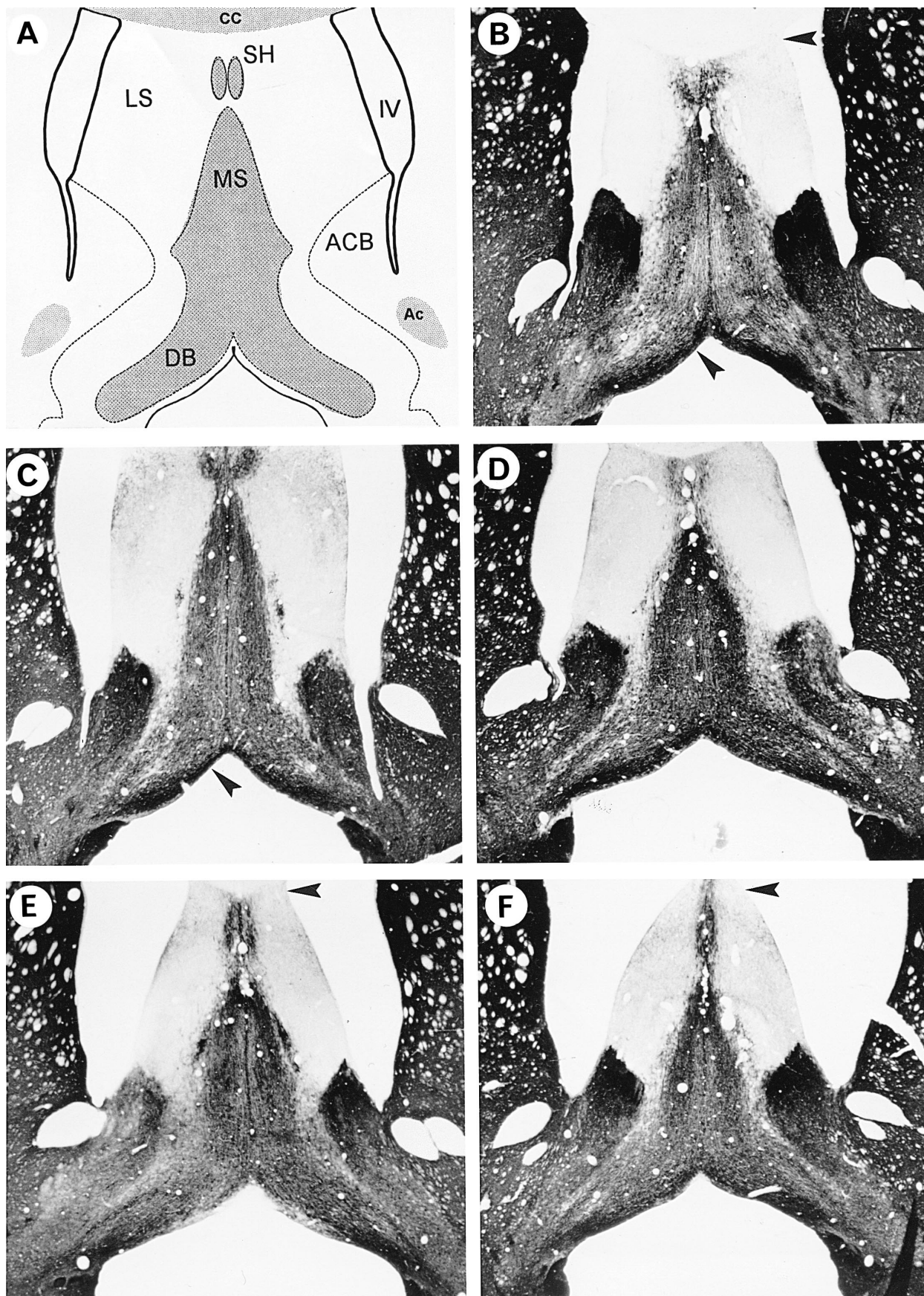
Insight into the neural regulation of social behaviour is still fragmentary. It might be expected that this pluriform phenomenon depend on the coordinated action of many brain regions. The limbic cortex, in particular the entorhinal region, occupies a top position in the hierarchy of information processing and is therefore presumed to be involved in regulation of the most complex functions, among which might be social behaviour. Our study supports this notion, showing relatively high correlation of a number of affected behavioural variables with measurements of the limbic cortex and MS-DB area. The latter region is intimately related to the entorhinal cortex and hippocampus, both anatomically and functionally [34], so that one might regard the effects of MAM as affecting cortical and subcortical components of the same associa-

tive limbic circuitry. It is noteworthy that the behavioural parameters in the significant correlations consist almost exclusively in measures of exploratory social behaviour. Whereas this might in part be due to the abundance of such behaviour relative to, for example, aggressive behaviour in Wistar rats, it may also reflect that the above mentioned structures are particularly involved in the regulation of this aspect of behaviour. It should in this context be considered that the present correlation analysis comprises only forebrain regions. Hence, it cannot be excluded that possible abnormalities in other parts of the brain may have contributed to the behavioural deficits observed in this study.

The role of the entorhinal cortex in spatial learning, although investigated repeatedly in view of its intricate connections with the hippocampus, is not yet altogether clear. Various studies have shown impaired reference memory after lesions of the entorhinal cortex, or perforant path, specifically in spatial paradigms, such as the Morris water maze [22,28] and eight-arm radial maze [27]. On the other hand, the acquisition of presumably simpler spatial tasks, requiring, for instance, the learning of one location, appears to be spared by such lesions [14,50]. Deficits in transient or 'working' memory have been reported more consistently after entorhinal cortex, or perforant path lesions. For instance, in spatial alternation tasks [50], radial mazes [49], delayed (non-) matching tasks [28] and in similar, non-spatial set-ups [38,48,50]. It should, hereby, be noted that entorhinal lesions in the above mentioned studies often encompass parts of the subicular complex, and/or Ammon's horn, which by themselves have been shown to induce spatial reference and working memory deficits [27,40].

Our own results indicate that both spatial reference and working memory can be sustained with a compromised lateral or medial entorhinal cortex. Although procedural differences exist between the above mentioned test paradigms and the one used by us, it seems justifiable to state that a developmental, proliferative insult to the entorhinal area does not produce the same memory deficits as do adult lesions. This may be due to the extent of entorhinal abnormalities following either procedure, to differential disconnection from surrounding structures, and finally, to different plastic consequences following insults at the prenatal and at the adult age. It might, furthermore, be noted that abnormalities of the septum and slight hypoplasia of the posterior hippocampus in MAM-treated animals were also insufficient to disrupt spatial learning.

In the hole board experiment, reduced working memory and increased time between hole visits, suggested a reduced performance efficiency on day five of the hole board test, in groups E10 and E11. This test day coincided with the weekly cleaning routine at the animal laboratories, which caused noises to penetrate in the animal stables and in the, otherwise silent, hole board environment. It appears that E10 and E11-treated animals may have reacted with



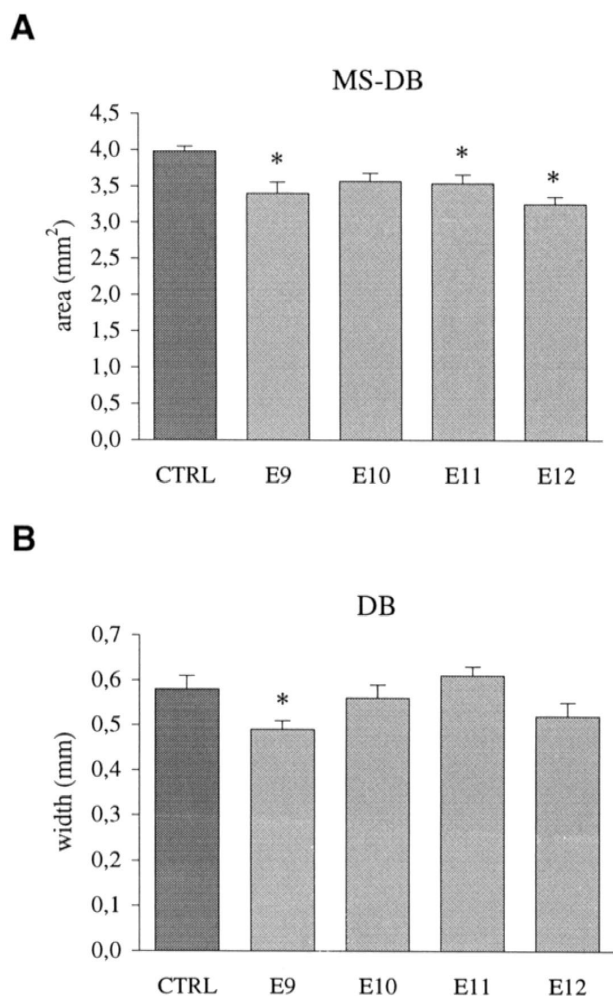


Fig. 9. Mean coronal surface area of the medial septum-diagonal band region (A) and width of the diagonal band region (B) following prenatal MAM administration on various days of embryonic development (E9–E12). * $p < 0.05$ in Mann–Whitney U -tests.

exaggerated sensitivity to these environmental disturbances. These observations might be related to our recent findings, showing impaired habituation to auditory startle stimuli in similarly treated rats [53,55]. Finally, E11-treated animals took significantly more time than controls to complete the hole board searches. In view of recent open field observations [20] this is not likely due to motor impairment. Further tests will be necessary to evaluate what other factors might have affected performance efficiency.

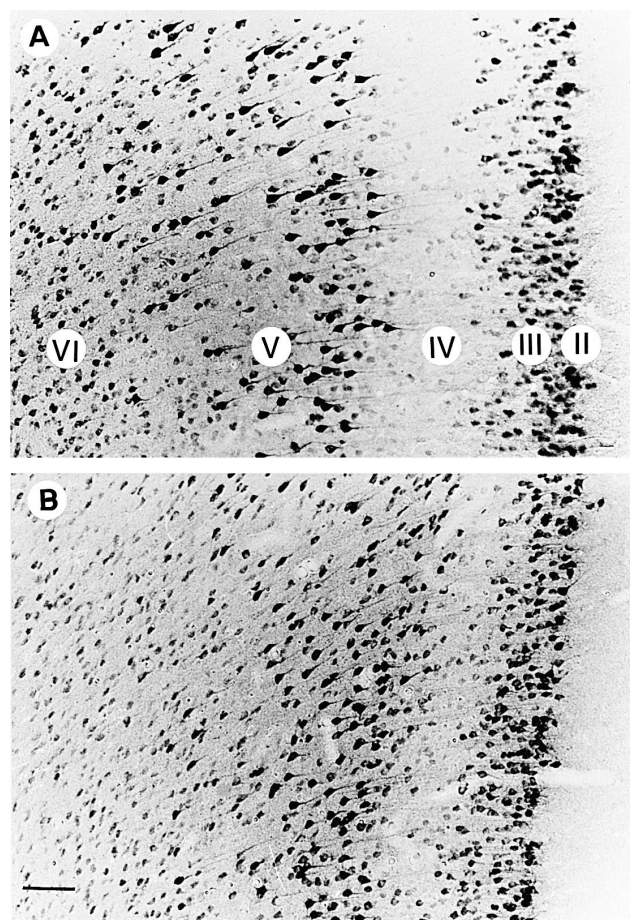


Fig. 10. Representative photomicrographs of PKC- γ immunostained sections, showing the anterior cingulate cortex of a control rat (A) and an E9-treated rat (B). Notice thinning of cortical layers and dense packing of pyramidal cells in (B). Bar: 175 μ m.

4.1. Implications for biological psychiatry

As known from a number of human developmental syndromes and also from experimental data [45], abnormalities at different stages of prenatal development can affect adult morphology and behaviour in different ways. Our own findings suggest that proliferative insults, during the mid-period of rat embryonic development, induce relatively subtle alterations of brain morphology in a number of closely interlinked limbic regions. These regions develop precociously in rodents [6,7] and also in men [31]. Notably, the pattern of morphological abnormalities induced by the prenatal insult with MAM to some extent

Fig. 8. Representative photomicrographs of acetylcholine esterase stained sections, showing the septal region of a control rat (B) and of MAM-treated rats from groups E9 to E12 (C–F). The position of various nuclei is schematically illustrated in (A). Notice that the width of the E9 diagonal band, between the arrow in C and the nucleus accumbens, is reduced with respect to control (arrow in B). Conversely, the medial septal complex, the septohippocampal nucleus (SH) and the width of the septum, especially underneath the corpus callosum (see arrows in B, E, F), are preferentially reduced in later treated animals. In addition, the rats exposed on E11 (E) and E12 (F) show striking enlargement of the lateral ventricles. Abbreviations: Ac, anterior commissure; cc, corpus callosum; DB, diagonal band; LS, lateral septum; MS-DB, medial septal complex; NAC, nucleus accumbens; SH, septohippocampal nucleus; IV, fourth ventricle. Bar: 700 μ m.

Table 3

	MEN	LEP	LEA	ECT	CIA	MSDB	HDB
<i>Wistar female</i>							
Social groom (f)	−0.60**						0.51*
Social groom (d)	−0.53*						
Investigate (f)		0.64**		−0.52*			
Investigate (d)		0.60**					
Non-social (f)			−0.51*				
<i>Wistar male</i>							
Social groom (f)					−0.51*		
Social groom (d)					−0.56*		
Genital sniff (d)							
<i>TMD S3 male</i>							
Agonism (f)						−0.58**	
Investigate (f)						−0.59**	

Abbreviations: MEN, medial entorhinal cortex; LEP, posterior lateral entorhinal cortex; LEA, anterior lateral entorhinal cortex; ECT, entorhinal cortex; CIA, anterior cingulate cortex; CIP, posterior cingulate cortex; MSDB, medial septum and diagonal band nucleus; HDB, horizontal limb of the diagonal band nucleus; (f), frequency; (d) duration.

* $p < 0.01$.

** $p < 0.01$.

parallels post mortem observations in schizophrenia. Indeed, several papers claim reduction of entorhinal cortex thickness [3,12], surface area [12], and disorganised cytoarchitecture [1,4,26] in schizophrenic subjects, while others report abnormal morphology of the anterior cingulate cortex (Ref. [8] and references therein) and hippocampus [3,24]. MAM administrations, as adopted here, moreover induce abnormal cerebral asymmetries [54], which have also been observed in schizophrenia [26,57].

At the behavioural level, impairments in social functioning are among the hallmarks of schizophrenia (DSM-IV; American Psychiatric Association, 1994) and are perhaps the strongest predictors of clinical outcome [56]. Specific deficits have been observed amongst others in facial affect- and social cue perception, social knowledge and social-cognitive problem solving [42], while the discourse of schizophrenics lacks referents and cohesive ties [44]. It is thought that such deficits stem from a tendency to make wrong inferences about the states of other people's minds [21]. We presently show that MAM-treated rats also show inadequate social behaviour, including decreased priming of behaviour by previous experience, which suggests impaired integration of incoming social stimuli with contextually relevant memory traces. Our findings suggest that this might be the consequence of faulty information processing in the entorhinal region and other limbic association areas, although influence from other putatively affected regions cannot be excluded. In these regions, particularly in the entorhinal cortex, information from all cortical channels ultimately converges. Hence, it can be envisaged that malfunction of these supramodal association cortices could produce abnormalities of association, cognition and attention with subsequent effects on behaviour. In line with this notion, deficits in sensory gating and attention have been observed in some schizophrenic subjects

[11,36] and also in rats with MAM-induced limbic cortex maldevelopment [53,55,52].

The combined findings in MAM-exposed rats support neurodevelopmental hypotheses of schizophrenia, showing that an insult of early cortical development can produce behavioural and brain morphological abnormalities that to some degree mimic observations in schizophrenic subjects. The abnormalities induced at this stage of development occur without concurrent gross teratology, or general impairment of higher brain functions, as suggested by the sparing of at least one such function: spatial learning.

Although the present investigations were instigated by findings in schizophrenia research, some of the social abnormalities induced by MAM administration bring to mind clinical observations in other presumably developmental psychiatric disorders. For example, some children with a, so-called, information processing disorder (DSM IV: developmental neurological disorder) display a behavioural pattern typecasted as 'active but odd'. These children are usually very active, display increased social initiative, and decreased social fear. The interpretation of social stimuli appears somewhat impaired, while there may be a tendency toward aggressive problem solving. Furthermore, in autism related disorders patients may present with strong reduction of the behavioural repertoire, sometimes with inappropriate introductory behaviour and severely impaired understanding of social stimuli. Interestingly, for some of these disorders research has implicated association areas in the temporal lobe and around the lateral sulcus, which also originate relatively early in CNS development [7,13].

In conclusion, the experimental disruption of limbic cortex development may present a useful approach to study the relationship between abnormal ontogenesis and schizophrenia. Similar strategies might be considered to

study other neuropsychiatric disorders with a presumed neurodevelopmental pathogenesis.

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